Induction of Protective Immunity by Synthetic *Vibrio cholerae* Hexasaccharide Derived from *V. cholerae* O1 Ogawa Lipopolysaccharide Bound to a Protein Carrier

Anatoly Chernyak,[1] Seiichi Kondo,[3] Terri K. Wade,[2] Michael D. Meeks,[2] Pedro M. Alzari,[4] Jean-Michel Fournier,[5] Ronald K. Taylor,[2] Pavol Kováč,[1] and William F. Wade[2]

[1]Laboratory of Medicinal Chemistry, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland; [2]Department of Microbiology and Immunology, Dartmouth Medical School, Lebanon, New Hampshire; [3]Department of Microbiology, School of Pharmaceutical Sciences, Josai University, Sakado, Japan; [4]Unité de Biochimie Structurale, Center National de la Recherche Scientifique, Unité de Recherche Associe 2185, and [5]Unité du Choléra et des Vibrions, Centre National de Référence des Vibrions et du Choléra, Institut Pasteur, Paris, France

Synthetic antigens that mimic the terminal hexasaccharide epitope of the O-specific polysaccharide of *Vibrio cholerae* O1, serotype Ogawa, were conjugated to bovine serum albumin (BSA). Conjugates with carbohydrate-to-carrier molar ratios of 15.5:1, 9.2:1, and 4.6:1 were tested for immunogenicity and efficacy in mice. The role of preimmunity to BSA and the use of adjuvant in the generation of the serologic response to the O-specific polysaccharide and protection against virulent *V. cholerae* was examined. Preimmunity to BSA did not affect the anti-Ogawa titers but seemed to enhance the protective capacity of antiserum. All 3 conjugates were immunogenic, but adjuvant was effective at inducing higher and earlier antibody responses. In tertiary serum samples, a correlation was found between vibriocidal activity and protection. The protective capacity of antiserum was evident in serum from mice immunized with all conjugates, but it was highest in the groups that received the conjugate with the lowest level of substitution. Further studies are required to increase understanding of the reason for differential protection.

In the continuing effort to develop a universally effective vaccine for cholera, it is of great importance that the new generation of vaccines be able to induce protective and memory responses to the bacterial lipopolysaccharide (LPS) in children. *Vibrio cholerae* O1 LPS has been shown to induce protective immune responses in humans and experimental animals [1–4], and thus its use as a protective immunogen for cholera vaccine development is widely accepted [5–7]. The O-specific polysaccharide (O-SP) of 2 *V. cholerae* serotypes, Ogawa and Inaba, which are the serotypes associated with epidemic cholera, consists of (1→2)-α–linked 4-amino-4,6-dideoxy-D-mannose (perosamine), the amino group of which is acylated with 3-deoxy-L-*glycero*–tetronic acid [8–11].

LPS is a type 1 T cell–independent antigen. At high levels, purified LPS is mitogenic for mouse B cells, resulting in panimmunoglobulin production. At low levels, LPS can induce LPS-specific IgM and IgG antibodies in humans and mice when the appropriate conditions are present [12, 13]. In the case of whole *V. cholerae*, LPS can induce isotype switching to IgG and IgA antibodies that are protective [4, 6, 7, 14]. The exact conditions needed for anti–O-SP antibody responses to form in humans with cholera have not been defined. Exposure to *V. cholerae* in the form of either infection or vaccination with intact bacteria can induce protective immunoglobulin characteristic of both T cell–independent responses (IgM) [15] and T cell–dependent responses (IgG and IgA) [4, 7, 15]. Identification of methods of experimentally manipulating the anti-LPS immune responses so that secretory IgA or IgG specific for *V. cholerae* O-SP are optimally induced will facilitate development of an effective cholera vaccine.

In an attempt to develop a vaccine that targets *V. cholerae* (O1 Ogawa or O139) O-SP without causing the complications that often arise from the use of native LPS as a vaccine, 2 groups have coupled "detoxified" LPS to protein carriers [1, 16]. The use of a synthetic, O-SP–based immunogen eliminates the toxicity problems associated with native LPS. The advantage of conjugation of a synthetic component of the Ogawa O-SP linked to a protein carrier, such as bovine serum albumin (BSA), is the opportunity to maximally induce a T cell–dependent immune response to LPS epitopes. T cell–dependent serologic responses

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Reprints or correspondence: Dr. William F. Wade, Dartmouth Medical School, Dept. of Microbiology and Immunology, 630W Borwell Bldg., Lebanon, NH 03755 (william.wade@dartmouth.edu).

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are characterized by early switching of the specific isotype from IgM to IgG (or IgA), affinity maturation of the antigen-combining site, and long-term B cell memory.

When the terminal sugar is removed from the LPS, the epitope resembles that of a type 2 T cell–independent antigen. Type 2 T cell–independent antigens have been engineered in association with protein carriers. Such conjugates have proven to be effective vaccines that are considered to be landmark advances in pediatric medicine for childhood diseases [17]. Crystal structures of the Ogawa monosaccharide or disaccharide complexed to an anti–Ogawa O-SP monoclonal antibody (MAb), S-20-4, helped to define the serologic specificity of the anti-Ogawa antibody [11]. This determinant is defined by a 2-*O*-methyl group on the nonreducing terminal perosamine unit, which has a free hydroxyl at the 2 position in the Inaba LPS [8, 18, 19]. The Ogawa epitope, when it is attached to a protein carrier, should also be immunogenic in children, who would not respond to the same carbohydrate epitopes if these epitopes were not coupled to protein carriers [20]. Recently, it was demonstrated that a synthetic oligosaccharide of *Streptococcus pneumoniae* type 3, conjugated to the CRM_{197} form of diphtheria toxin [21], could induce protective IgG responses in mice.

The identification of the structure (Ogawa epitope) with the highest binding affinity for the protective MAb S-20-4 [9] and the recent development of the chemistry [22] required to link the Ogawa epitope as defined by the S-20-4 MAb has allowed the development of such a synthetic Ogawa immunogen [23]. We hypothesize that a neoglycoconjugate immunogen produced by conjugation of the LPS-derived Ogawa epitopes (CHO) to a protein carrier (BSA) will be immunogenic and will induce protective T cell–dependent antibodies. We incorporated elements into the experimental design that would test the role of preexisting immunity to the carrier and the effect of adjuvant on the subsequent humoral responses to the CHO-BSA conjugates. These conjugates differ in the molar ratio of the linker-equipped hexasaccharide to BSA.

Materials and Methods

Animals. Six-week-old female BALB/c mice were purchased from the National Cancer Institute (Bethesda, MD) and used as hosts in our immunization studies. Untimed pregnant CD-1 females were purchased from Charles River Breeding Laboratories and allowed to deliver the pups, which were used at 4–5 days of age in the infant mouse protection assay. All mice were housed under standard conditions in the Animal Resources Center located at the Dartmouth-Hitchcock Medical Center (Lebanon, NH) and maintained on a basic diet of Harlan Teklab sterilizable rodent feed.

Immunization and serum collection. Twelve groups of 4 mice each were used to test the immunogenicity of the Ogawa O-SP epitope conjugates, which were variably substituted with the Ogawa terminal hexasaccharide in the following molar ratios of CHO to BSA: conjugate A, 15.5:1; conjugate B, 9.2:1; and conjugate C, 4.6:1. We preimmunized some groups of mice to BSA, the carrier for the CHO, to determine whether preexisting antibody or carrier-specific T cells would affect subsequent immune responses to the CHO-BSA. Mice in treatment groups 1, 3, 5, 7, 9, and 11 were preimmunized with BSA (25 μg/mouse) and alum (Pierce Chemical) and 2.5 μg of cholera toxin (CT; Sigma-Aldrich) via intraperitoneal injection (200 μL/mouse) 14 days before the start of the CHO-BSA immunization regimen and again on day +15 (figure 1). Groups 2, 4, 6, 8, and 10 received alum and CT without BSA to normalize the response to alum and CT, which were part of the immunization regimen for inducing preimmunity to the carrier. Alum and CT were chosen to deliver the BSA in an attempt to skew the T helper cell response toward IgA. Preimmunization serum samples were collected 3 days before the primary immunization with CHO-BSA. Mice were given 10 μg (based on carbohydrate weight) of CHO-BSA conjugate intraperitoneally on days 0, +14, and +24, except for mice who were to receive conjugate C; those mice received only the first 2 inoculations, because there was a limited amount of conjugate C available when we began the experiment. Groups 3, 4, 7, 8, 11, and 12 received an emulsion of the CHO-BSA conjugate and RIBI adjuvant (Corixa); all other groups were immunized with conjugate in saline. RIBI adjuvant, which contains highly refined, nontoxic monophosphoryl lipid A and synthetic trehalose dicorynomycolates, an analogue of trehalose dimycolate from the cord factor of the tubercle bacillus, was mixed with the CHO-BSA conjugates. Blood was collected under light isoflurane anesthesia via the retroorbital sinus/plexus on days

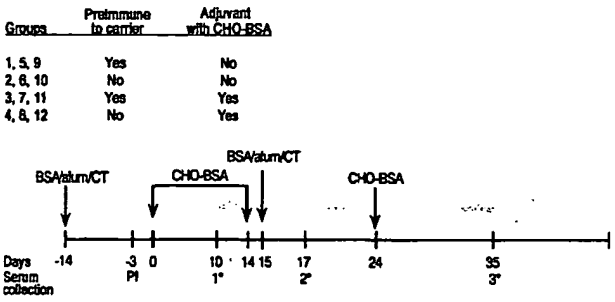


Figure 1. Time line for immunization and blood sampling of various groups of 4 mice in a study of protective immunity to cholera. Mice in groups 1–8 received immunizations on days 0, +14, and +24. Mice in groups 9–12 received immunizations on days 0 and +14 only. Mice in all groups except 1, 2, 5, 6, 9, and 10 received neoglycoconjugate immunogen (CHO [Ogawa terminal hexasaccharide]–bovine serum albumin [BSA]) in adjuvant. Mice in groups 1, 3, 5, 7, 9, and 11 were preimmunized with BSA/alum/cholera toxin (CT) on day −14 and day +15; mice in other groups (2, 4, 6, 8, 10, and 12) received alum and CT on those days. The effect of adjuvant (groups 3, 4, 7, 8, 11, and 12) on immunogenicity of CHO-BSA vs. immunogenicity of CHO-BSA alone (groups 1, 2, 5, 6, 9, and 10) was tested. Mice were immunized with BSA carrier to test the effect of anti-BSA antibodies and T cells on the induction of anti-CHO responses by CHO-BSA conjugates. Immunization with BSA was accompanied by alum and CT to skew immune responses toward a Th2 phenotype. Serum samples were collected on days −3 (preimmunization serum sample; PI), +10 (primary serum sample; 1°), +17 (secondary serum sample; 2°), and +35 (tertiary serum sample; 3°).

+10, +17, and +35, which represent primary, secondary, and tertiary serum samples, respectively. The blood was incubated for 30 min at 37°C and then overnight at 4°C, after which serum was collected and 20–25-μL aliquots were stored at 4°C and −20°C until use.

Female BALB/c mice were used to test immune responses to whole *V. cholerae* O1 Ogawa LPS. Blood was drawn from 5 mice on day 0, and the mice were then inoculated with LPS Ogawa NIH-41 (9 μg/mouse intraperitoneally). Booster immunizations were given on days +10 and +19. Primary, secondary, and tertiary serum samples were collected on days +10, +19, and +26, respectively.

CHO-BSA conjugates. Immunogens (figure 2) were prepared by linking of BSA to the chemically synthesized, linker-equipped hexasaccharide fragment of the O-SP of *V. cholerae* O1 Ogawa by use of squaric acid chemistry [23–25]. In brief, condensation [22] of the glycosyl acceptor (a 2-trimethylsilyl glycoside with chemical properties that allow the aglycon to be exchanged at the desired synthetic stage; figure 2*B*, *2*) and glycosyl donor *(3)*, prepared [26] from methyl 4-azido-3-*O*-benzyl-4,6-dideoxy-α-D-mannopyranoside [27–29], gave disaccharide *(4)*, which was transformed [22] into synthons *(5* and *6)*. Coupling of these yielded a tetrasaccharide with a selectively removable *O*-acetyl group at the terminal glycosyl residue. Deacetylation of the tetrasaccharide, followed by condensation of the product with the disaccharide glycosyl donor *(5)*, yielded a hexasaccharide with, again, a selectively removable *O*-acetyl group at the terminal glycosyl residue. After deacetylation and methylation of the hydroxyl group thus liberated, to position the methyl group at position 2 of the terminal dideoxymannosyl residue, as is characteristic of the Ogawa serotype, the hexa-azide was selectively reduced with hydrogen sulfide [27] to get the hexa-amine *(7)*. The chiral N-side chain that is characteristic of the O-SP of *V. cholerae* O1 was introduced by carbodiimide-mediated amidation, with use

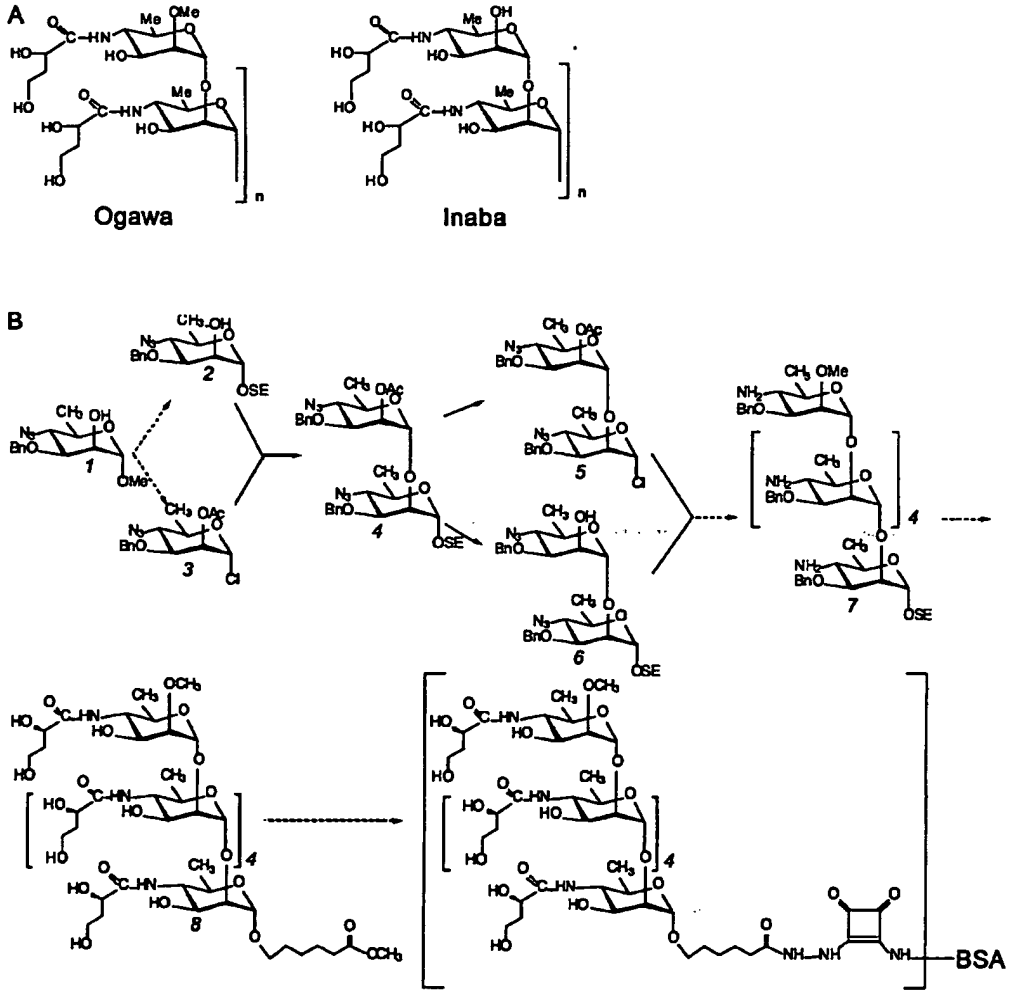


Figure 2. Structure of O-specific polysaccharide of *Vibrio cholerae* O1, serotypes Ogawa and Inaba. *A*, Difference between terminal sugars on Inaba and Ogawa lipopolysaccharide. Structures differ only in the presence of a terminal 2-*O*-methyl group (*left*, OMe; *right*, OH). *B*, Schema for generation of neoglycoconjugate immunogen (CHO [Ogawa terminal hexasaccharide]–bovine serum albumin [BSA]). *Solid arrows*, 1 step; *dotted arrows*, multiple steps. Ac, acetyl; Bn, benzyl; Me, methyl; SE, 2-trimethylsilyl ethyl.

of 4-*O*-benzyl-3-deoxy-L-*glycero*-tetronic acid [30] as the acylating reagent. After use of multistep aglycon exchange to introduce the methyl 6-hydroxyhexanoate spacer, hydrogenolytic debenzylation afforded the deprotected linker-equipped hexasaccharide fragment of the O-SP [22]. Hydrazinolysis of *(8)* gave the corresponding hydrazide, which was treated with squaric acid dimethyl ester as required by the squaric acid chemistry conjugation protocol [24–26]. Samples were periodically withdrawn from the conjugation mixture and analyzed by surface-enhanced laser desorption/ionization time-of-flight mass spectrometry. This allowed termination of the reaction when the desired loading of the hapten onto the carrier protein had been achieved [23]. The obtained neoglycoconjugates, A, B, and C, contained, on average, 15.6, 9.2, and 4.6 mol hapten per 1 mol BSA, respectively.

Serologic testing. The presence of anti–Ogawa O-SP antibodies in individual serum samples was measured by ELISA. Semipurified Ogawa LPS from *V. cholerae* strain NIH-41 was used as test antigen [31]. High-binding, 96-well, flat-bottomed microtiter plates (Corning Life Sciences) were coated with 5 μg of Ogawa LPS in 100 μL of 0.1 *M* carbonate-bicarbonate buffer (pH 9.5) and incubated overnight at 4°C. Plates were washed twice with a Skan Washer 400 microplate washer (Molecular Devices) with 250 μL of 1× PBS–0.05% Tween 20 (Fisher Scientific). Nonspecific binding was blocked with 200 μL of buffer (consisting of 1× PBS, 1.0% fish gelatin [BioFX], 1.0% normal goat serum, and 0.05% Tween 20) for 2 h at 37°C. Plates were washed twice more, after which 50 μL of antiserum (serially diluted 2-fold) was added to each well and incubated overnight at 4°C. The initial dilution of 1:200 was used for all serum samples. Plates were then washed twice, and 50 μL of horseradish peroxidase–labeled goat anti–mouse IgM (μ chain specific; Southern Biotechnology Associates) or IgG (heavy chain and light chain specific; Kirkegaard & Perry Laboratories) detector antibody (diluted 1:4000) was added to each well and incubated at 37°C for 1 h in the dark. Plates were washed twice and developed with 100 μL of *o*-phenylenediamine dihydrochloride (OPD) peroxidase substrate for 10 min at room temperature. OPD peroxidase substrate was prepared by dilution of 10-mg tablets in 0.05 *M* phosphate-citrate buffer (pH 5.0) to a final concentration of 0.4 mg/mL. Fresh 30% H_2O_2 was added to the OPD immediately before use, to a final concentration of 0.02%. The reaction was stopped with an equal volume of 3 *M* HCl. Optical densities were read at 490 nm by use of a Dynex Technologies MRX microplate reader (Thermo Labsystems) and Dynex Revelation version 3.04 software.

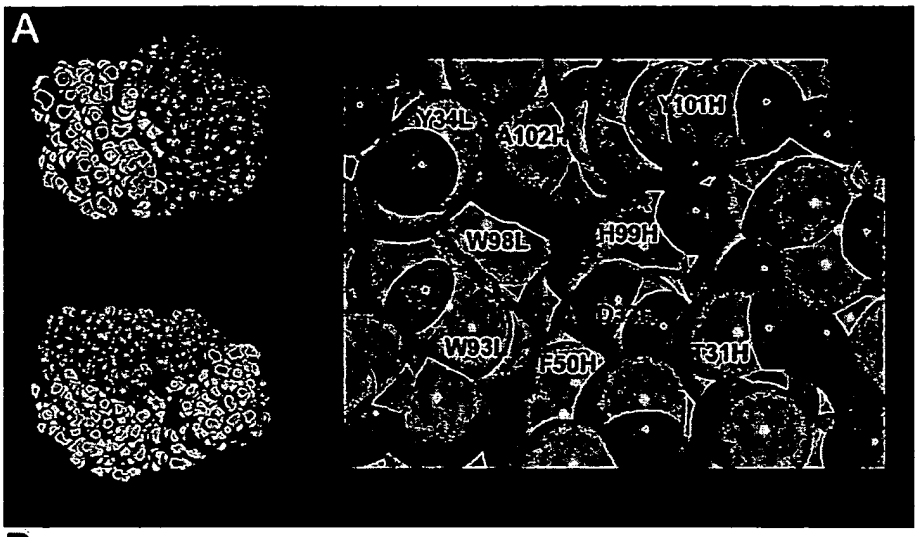
All calculations and statistical analyses were done with Microsoft Excel 9.0 and GraphPad Prism 3.0. Student's *t* test or analysis of variance was used to compare mean titers of tertiary serum samples. We compared the tertiary serum sample titers because they were the source of serum for the functional assays. Correlation coefficients were also calculated as appropriate. End-point titers for ELISA were defined as the reciprocal of the dilution for the last positive well for each sample after the background value had been subtracted. Background values were determined with use of preimmunization serum samples. One to 3 different individual preimmunization serum samples were analyzed on multiple 96-well plates. The optical densities of the highest dilution of the preimmunization serum samples were averaged and then multiplied by 2. This value was subtracted from the optical densities of all of the wells containing the titration of the individual serum samples. After this manipulation, the titer in the final positive well was considered to be the end-point titer.

The concentration of total IgG specific to Ogawa O-SP in the pooled tertiary antiserum from each treatment group was determined by ELISA. Equal volumes of purified mouse IgG1, IgG2a, IgG2b, and IgG3 isotype controls (Southern Biotechnology Associates) were pooled and assessed by Bradford assay (Bio-Rad) for total protein concentration against a known total IgG standard (Sigma-Aldrich) and a known BSA standard (Pierce Chemical). A standard curve for total IgG (all subclasses) was generated by ELISA, using a series of 8 dilutions (5-fold each) of a stock 1-mg/mL solution of pooled mouse IgG subclasses. Duplicate samples of pooled tertiary serum from each treatment group were diluted 1:100 and 1:1000 in a 96-well plate and reacted against Ogawa LPS as described in the section "Serologic testing." Averaged optical densities from each test sample were used to calculate the total concentration of IgG specific to Ogawa in pooled tertiary serum on the basis of values in the standard curve.

Vibriocidal antibody inhibition. Vibriocidal antibody titers to *V. cholerae* (classic strain O395) were assessed by microdilution assay in vitro [32, 33]. Bacteria were grown in Luria-Bertani broth at 37°C for 18 h. The culture was centrifuged for 10 min at 180 *g*, resuspended into an equal volume of 1× PBS with 0.1% peptone (PBS-peptone), and diluted $1:1.0 \times 10^4$ in PBS-peptone. Pooled preimmunization and tertiary serum samples from each treatment group were diluted in 50 μL of ice-cold 1× PBS and 20% guinea pig complement (Sigma) at $1:1.0 \times 10^2$, $1:5.0 \times 10^2$, $1:1.0 \times 10^3$, $1:1.0 \times 10^4$, and $1:5.0 \times 10^4$ and kept in an ice-water bath until needed. Bacteria, representing 5×10^7 cfu, were mixed with diluted antiserum 1:1 and incubated for 1 h on a platform shaker at 37°C (250 rpm) before being returned to the ice-water bath. Each sample (total volume, 100 μL) was then spread on Luria-Bertani agar plates and allowed to dry completely at room temperature before overnight incubation at 37°C. Colony-forming units were recorded for each plate. Inhibition of bacterial growth (end-point titer) provided by the antiserum was considered to be significant if ≥50% of the bacteria were killed, compared with the numbers of colony-forming units from plates containing preimmunization serum, complement, and input bacteria.

Infant mouse challenge. The infant mouse challenge model for cholera was used to assess the protective quality of anti–Ogawa O-SP antibodies in vivo [34]. Cultures of *V. cholerae* O395 (classic Ogawa serotype) were grown for 18 h under toxin-coregulated pilus–expressing conditions (Luria-Bertani agar, pH 6.5, at 30°C). Twenty-five microliters of bacterial inoculum, representing 29–43 LD_{50}, were combined with 25 μL of PBS, positive control MAb A-20-6, or pooled tertiary antiserum immediately before intragastric administration to 4–5-day-old CD-1 mice. Challenged mice were kept at 30°C and monitored every 4 h, beginning 24 h after challenge, until the termination of the assay at 52 h. Survival curves were compared using the log-rank analysis available in GraphPad Prism 3.0.

Modeling of Ogawa terminal CHO epitope complexed with anti-Ogawa MAb S-20-4. The sequences of the 3 anti-Ogawa MAbs (figure 3) have been published elsewhere, as has the crystal structure for one of those antibodies bound to the terminal sugar residue of the Ogawa LPS [9, 11]. The Brookhaven Protein Data Bank (PDB; available through the National Center for Biotechnology



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RESIDUE   1        10        20        30        40        50
          |        |         |         |         |         |
2D6       DVKLVESGGGLVKPGGSLKLSCAASGFAFSSYDISWFRQTPERRLEWVAF
A-20-6    E-Q-E-------T-----R--------V--T-DM--V-----------F
S-20-4    E-Q-E-------T-----R--------V--T-DM--V-----K------F
VH7183.14 E-K-------------------------S-DM--V-----K------T
VH81X     E-Q---------Q-RE------ESNE-E-PSHDM--V-K--K------A

                                                  V D(N?)J
RESIDUE    52a      60        70        80  82abc     90  95    100
           |        |         |         |           |   |     |
2D6       ITSGGGGTSYPDTVKGRFTISRDNAKNILYLQMSSLKSEDTAMYYCARHYYGYLDYWGQGTTLTVSS
A-20-6    -S---SR-S-----------------------N-QS---------T-H--AV-----R--------
S-20-4    -S---R-S-----------D-------------Q----------T-HF-AV-----R--------
VH7183.14 -S---Y-Y-----------------R-----------R-----L-------
VH81X     -N-D-GS-Y----MER--I-----T-KT--------R----------
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Figure 3. *A,* Crystallographic structure of anti–*Vibrio cholerae* Ogawa O-specific polysaccharide, S-20-4, showing a patch of hydrophobic side-chain residues at the center of the antibody interface [11]. *Left,* Heavy chain *(dark blue)* and light chain *(light blue)* of S-20-4, with the hydrophobic patch highlighted in gold. *Right,* Hydrophobic patch, formed by residues from both light (Tyr34L, Trp93L, Trp98L) and heavy (Phe50H, Tyr101H, Ala102H) chains. Together, they define a nonpolar pocket that accommodates the 2-*O*-methyl group of the terminal perosamine sugar [9, 11]. We hypothesize that Phe50H and Ser59H (not shown) were selected by the antigen and are required for stabilization of anti-Ogawa antibody–based 81X or 7183.14 V_H7183 family members. The RasMol program (Biomolecular Structure Department, Glaxo Wellcome Research and Development, Greenford, UK) was used to display and manipulate Brookhaven Protein Data Bank (available through the National Center for Biotechnology Information [http://www.ncbi.nlm.nih.gov]) file 1F4Y to generate this figure. *B,* Comparison of selected translated V heavy-chain 7183 family members to anti-Ogawa monoclonal antibody V heavy-chain cDNA sequences. Primary amino acid sequences (Kabat numbering system) of 3 anti-Ogawa monoclonal antibodies (2D6 [IgA], A-20-6 [IgG], and S-20-4 [IgG]) are shown in relation to 2 translated germline sequences from 7183.14 and 81X V_H7183 family members [35, 36]. Amino acids noted in the crystal structure *(A)* and printed in large red type *(B)* are important to the interaction of S-20-6 and Ogawa epitopes. D, diversity; J, joining; N, nucleotide region; V, variable.

Information [http://www.ncbi.nlm.nih.gov]) file 1F4Y was manipulated with the RasMol program (R. Sayle and Biomolecular Structure Department, Glaxo Wellcome Research and Development, Greenford, UK) to generate figure 3. The numbers we used to define the position of amino acids on the crystal are from the PDB file; those used to discuss the amino acid sequences of the 3 anti-Ogawa MAbs [9] were based on the Kabat numbering system. The description of the interaction of the terminal sugar and the Fab of S-20-4 also used the Kabat numbering system. However, the PDB file does not recognize numbers with letters after them. Thus, the use of 52a, 82a, 82b, and 82c (Kabat numbers) results in a difference in the PDB file of 4 positions that define the amino acid numbers. The amino acids are shown in 3-letter code. In our discussion, Ser59 (crystal) is Ser58 (Kabat), His99 (crystal) is His95 (Kabat), and Ala102 (crystal) is Ala98 (Kabat), as described by Villeneuve et al. [11], who used the Kabat numbering system.

Source of immunoglobulin heavy-chain variable region sequences. The germline heavy-chain variable region sequences in figure 3 were derived from translated nucleotide sequences that have been reported elsewhere [35, 36].

Results

The vast majority of epidemic cholera is caused by *V. cholerae* strains of the O1 serogroup, which is subdivided into 2 serotypes, Ogawa and Inaba. MAbs have been generated to epitopes on the LPS of both Ogawa and Inaba [7, 37]. The MAbs S-20-4, A-20-6 (IgG1), and 2D6 (IgA) define a serologic epitope of Ogawa that is not present in Inaba. The protective epitope that defines the Ogawa serotype of LPS has been characterized serologically [9] and by radiographic crystallographic analysis [11]. The epitope has been shown to depend on a single 2-*O*-methyl group on the nonreducing terminal perosamine unit of the Ogawa O-SP (figure 2*A*). Recently, Ogawa et al. [22] synthesized this epitope in a linker-equipped form, and Chernyak et al. [23] coupled it to a carrier protein. In this study, we tested 3 different immunogens based on the synthetic Ogawa epitope that varied in the number of hexasaccharide residues and were covalently coupled to BSA by use of the reaction sequence shown in figure 2*B*.

Bacterial LPS is a type 1 T cell–independent antigen. The intrinsic mitogenic activity of LPS activates B cells to produce LPS-specific antibody without direct T cell help but with contributions of cytokines from other immune effectors [12]. Experimentally induced anti-cholera LPS responses to purified LPS and/or to LPS conjugates are typically of the IgM isotype, and lower concentrations of IgG also are present after infection or immunization [1, 16]. However, after hyperimmunization, anti-LPS IgG and IgA can be generated [7, 37]. In our study, mice immunized intraperitoneally, 3 times, 9–10 days apart, with 9 μg of whole Ogawa LPS generated anti-LPS IgM responses after an intraperitoneal immunization. The IgM response improved slightly when a booster intraperitoneal injection was administered, but the IgG response to LPS immunization remained low after 3 immunizations, ranging from ≤1:200 to 1:400 (figure 4).

The vigorous anti–Ogawa O-SP IgM and IgG responses (figure 5) made by mice immunized with CHO-BSA conjugates A–C contrasted with findings for mice immunized intraperitoneally with whole Ogawa LPS (figure 4). IgG responses clearly were more effectively induced by CHO-BSA, and although IgM responses could be seen in mice immunized with LPS or CHO-BSA, the strength of the response was greater in the CHO-BSA–immunized mice, which suggests that the mice had a more uniform response to CHO-BSA cholera epitopes than to those presented by LPS. The experiments depicted in figure 5 were designed to test the role of existing immunity to the carrier and the effect of adjuvant combined with CHO-BSA on anti–O-SP serologic responses.

Adjuvant effects. Immunization with adjuvant is not required for generation of anti–CHO-BSA IgG or IgM responses. However, the inclusion of adjuvant clearly induced higher titers in serum and a response was seen at an earlier point than when CHO-BSA immunogen was used alone (figure 5; compare groups 1 and 2 [no adjuvant] with groups 3 and 4 [adjuvant], 5 and 6 with 7 and 8, and 9 and 10 with 11 and 12). In general, IgG responses could be found in the primary serum samples of mice that received the CHO-BSA in adjuvant. These responses increased with time and were, on average, 1:2500 in the tertiary serum samples. In contrast, the only IgG responses that were detectable in mice that received CHO-BSA without adjuvant were in the tertiary serum samples and typically were <1:1000.

CHO-BSA conjugates induced an IgM response that could be measured in primary serum samples (figure 5*A*). This response was most readily seen in mice that received the CHO-BSA with adjuvant. As was true of the anti–Ogawa O-SP IgG response to adjuvant, the IgM titers increased with time throughout the immunization course and were highest in the day +35 (tertiary) serum samples. In contrast, the IgM response of mice immunized with CHO-BSA

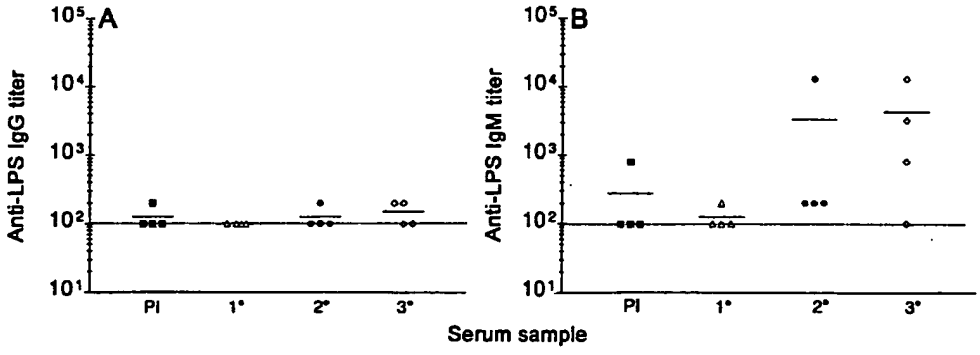

Figure 4. IgG *(A)* and IgM *(B)* responses to *Vibrio cholerae* Ogawa lipopolysaccharide (LPS) after intraperitoneal immunization of mice with 9 μg of whole LPS. Mice were immunized 3 times at weekly intervals (days 0, +10, and +19), and blood was collected on days 0 (preimmunization serum samples [PI]; *filled squares*), +10 (primary serum samples [1°]; *open triangles*), +19 (secondary serum samples [2°]; *filled circles*), and +26 (tertiary serum samples [3°]; *open diamonds*). Horizontal lines indicate mean titers of groups; symbols represent individual mice. Dotted lines indicate starting dilution of antiserum used in the ELISA.

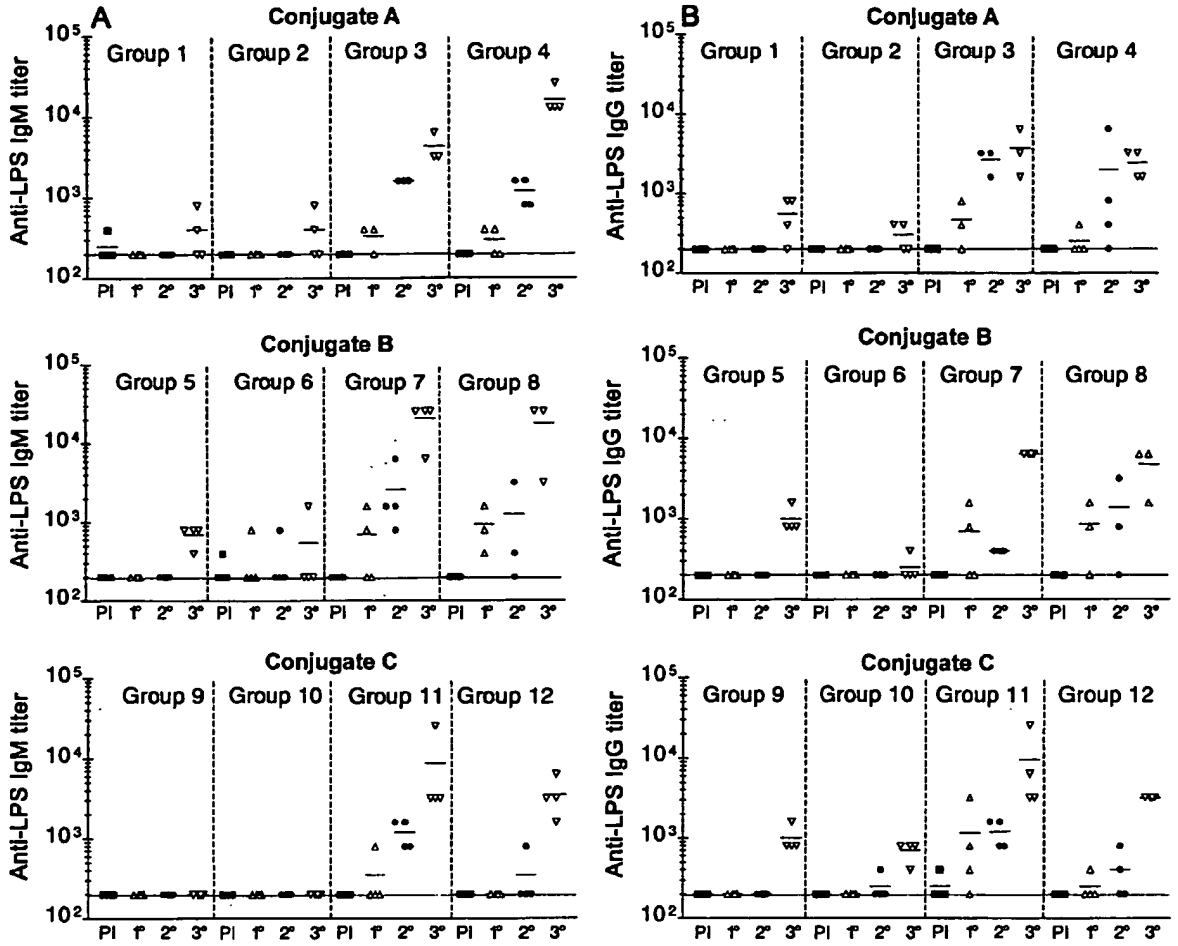

Figure 5. IgM *(A)* and IgG *(B)* responses to *Vibrio cholerae* Ogawa lipopolysaccharide (LPS) after intraperitoneal immunization of mice with CHO–bovine serum albumin (BSA) conjugates (LPS-derived Ogawa epitopes [CHO] conjugated to BSA). The immunization schedule is shown in figure 1. For a description of conjugates A, B, and C, see the section "CHO-BSA conjugates" in Materials and Methods. Horizontal lines indicate mean titers of groups; symbols represent individual mice. Dotted lines indicate the starting dilution of antiserum used in the ELISA. Serum samples were collected on days −3 (preimmunization serum samples [PI]; *filled squares*), +10 (primary serum samples [1°]; *open triangles*), +17 (secondary serum samples [2°]; *filled circles*), and +35 (tertiary serum samples [3°]; *inverted open triangles*). Mean ELISA titers of tertiary antiserum samples for all groups were calculated and used to generate *A* and *B* in figure 7. Comparison of mean titer of tertiary serum for group 3 vs. group 4, $P = .23$; for group 7 vs. group 8, $P = .42$; for group 11 vs. group 12, $P = .32$ (Student's t test); for comparison of all possible combinations, $P > .05$ (analysis of variance).

alone was detectable only in 4 of 6 groups at day +35, and it was similar in magnitude, at that point, to the IgM response at day +10 (primary serum samples) of mice immunized with CHO-BSA in adjuvant. Clearly, the use of adjuvant to deliver the immunogen results in higher and earlier responses than are seen when immunogen is delivered without adjuvant.

Preimmunity to carrier protein. Selected groups (1, 3, 5, 7, 9, and 11) of mice were immunized with BSA in alum accompanied by CT, in an attempt to induce both B cell and T cell immunity to the carrier before immunization with CHO-BSA. We wanted to test whether existing memory T cells specific to the carrier or existing carrier-specific antibody that could complex with immunogen would be a mitigating factor in the immune response of mice to the CHO-BSA immunogen. At day −3, mice that had been immunized with BSA at day −14 had significant anti-BSA responses, and mice that were not preimmunized with BSA had baseline titers (data not shown). Preimmunized mice, however, did not respond to the CHO epitope in a manner different from that of mice that were not preimmunized with BSA (figure 5) (comparison of mean tertiary serum titers for group 3 vs. group 4 yielded $P = .23$; for group 7 vs. group 8, $P = .42$; and for group 11 vs. group 12, $P = .32$ [Student's t test]; for all possible combinations, $P > .05$ [analysis of variance]). The difference in the slopes of the lines showing the increase in anti–

Table 1. Vibriocidal titers of serum samples from mice immunized with *Vibrio cholerae* O1 Ogawa CHO-BSA (lipopolysaccharide-derived Ogawa epitopes conjugated to a protein carrier).

CHO-BSA conjugate, group	Type of immunization received		Vibriocidal titers in serum	
	Preimmunization	With adjuvant	Preimmunization samples	Tertiary samples
A				
1	Yes	No	<100	1000
2	No	No	<100	500
3	Yes	Yes	<100	<100
4	No	Yes	<100	5×10^4
B				
5	Yes	No	<100	1×10^4
6	No	No	<100	<100
7	Yes	Yes	<100	5×10^4
8	No	Yes	<100	<100
C				
9	Yes	No	<100	$\geq 5 \times 10^4$
10	No	No	<100	1×10^4
11	Yes	Yes	<100	$\geq 5 \times 10^4$
12	No	Yes	<100	$\geq 5 \times 10^4$

NOTE. For a description of conjugates A, B, and C, see the section "CHO-BSA conjugates" in Materials and Methods. Pooled preimmunization (day −3) or tertiary antiserum (day +35) was combined with PBS, 20% guinea pig complement, and 5.0×10^7 cfu of *V. cholerae* strain O395. After 1 h of incubation, inoculum was spread on Luria-Bertani plates for overnight incubation at 37°C and counting of colonies. Tertiary antiserum values were used to generate *C* of figure 7. BSA, bovine serum albumin.

Ogawa O-SP titers over time was most notable for groups 11 and 12, but too few data points are available for a statistical comparison of the slopes of the lines for groups 11 and 12 with those for the other groups to be possible.

In vitro killing of V. cholerae. Vibriocidal assays are a standard measure for prediction of the protective nature of cholera antiserum [3, 14, 16]. We assessed the vibriocidal activity of pooled tertiary serum samples from the various groups of mice (table 1). Preimmunization serum samples were universally ineffective at killing input bacteria. The vibriocidal titer of the tertiary antiserum ranged from <100 (groups 3, 6, and 8) to ≥50,000 (groups 4, 5, 7, 9, 11, and 12). As a group, the mice immunized with conjugate C (molar ratio of CHO to BSA, 4.6:1) generated consistently higher vibriocidal titers than did mice immunized with conjugate A or B, which had higher levels of CHO substitution (molar ratios of CHO to BHA of 15.5:1 and 9.2:1, respectively). The nonvibriocidal responses of groups 3, 6, and 8 were reproducibly negative in the vibriocidal assay.

In vivo protection against virulent V. cholerae. The infant mouse assay is the current standard for evaluation of the efficacy of cholera antiserum in vivo [38]. We assessed pooled tertiary serum samples from all groups for the ability to protect against 29–43 LD_{50} of virulent *V. cholerae* Ogawa (figure 6). The protection at 52 h after inoculation of a 1:1 mix of antiserum and bacteria ranged from 10% to 100%. The MAb A-20-6, developed by Bougoudogo et al. [37], was used as a positive control and protected 100% of the infant mice over the course of the experiment. The negative control group (mice that received PBS mixed with bacteria) had >50% mortality at 24 h and 100% mortality at 52 h. Antiserum from groups 1–8 was variably protective (range, 10%–80%; mean, 35.7%). In these groups, in which mice received either conjugate A (molar ratio of CHO to BSA, 15.5:1) or conjugate B (molar ratio of CHO to BSA, 9.2:1), there was no clear pattern of protection afforded by preimmunity to either the carrier or adjuvant. Although the pooled antiserum from groups 1–8 was not completely ineffective (average protection for all groups receiving conjugate A, 34%, or B, 43%), it was not as protective as the antiserum from groups 9–12, which was derived from mice inoculated with conjugate C (molar ratio of CHO to BSA, 4.6:1; average protection, 70%). A statistical comparison (log-rank test) of the survival curves demonstrated that the protection associated with immunization of mice with conjugate C was significantly greater than that associated with conjugate A or conjugate B (conjugate A vs. conjugate B, $P = .17$; conjugate A vs. conjugate C, $P < .0001$; conjugate B vs. conjugate C, $P = .002$). The statistical analysis of whether preimmunity to the carrier affected survival was evaluated by comparison of pooled data for groups 2 and 4, 6 and 8, and 10 and 12, which represent the protection in serum from mice that did not receive prior immunization with BSA, with data for groups 1 and 3, 5 and 7, and 9 and 11, in which the mice received prior BSA immunization. In these groups, preimmunity to the carrier appeared to make a difference in protection (for groups 2 and 4 vs. groups 1 and 3, $P = .05$; for groups 6 and 8 vs. groups 5 and 7, $P = .001$; and for groups 10 and 12 vs. groups 9 and 11, $P = .04$). The statistical analysis of the effect of adjuvant use on survival was evaluated by comparison of pooled data for groups 1 and 2, 5 and 6, and 9 and 10, which represent the protection in serum from mice that did not receive adjuvant with the CHO-BSA, with data for groups 3 and 4, 7 and 8, and 11 and 12, in which mice received adjuvant with the CHO-BSA. The use of adjuvant did not induce a more consistent level of protection (for groups 1 and 2 vs. groups 3 and 4, $P = .53$; for groups 5 and 6 vs. groups 7 and 8, $P = .56$; and for groups 9 and 10 vs. groups 11 and 12, $P = .13$).

Predictor of protection. To determine which of the parameters we measured best predicted the protection measured in the infant mouse assay, we compared the tertiary serum sample data for the ELISA titers for anti–Ogawa O-SP IgM and IgG, vibriocidal titers, and total serum IgG concentration with the percentage of protection provided by pooled serum samples (figure 7). All 4 analyses indicated that pooled serum from mice inoculated with conjugate C afforded the most effective protection, but only vibriocidal titers clearly correlated with protection (figure 7*C*). In this analysis, the results were segregated into 2 groups: vibriocidal titers of <1:1000 resulted in ≤30% protection, and vibriocidal titers of ≥1:10,000 resulted in ≥50% protection.

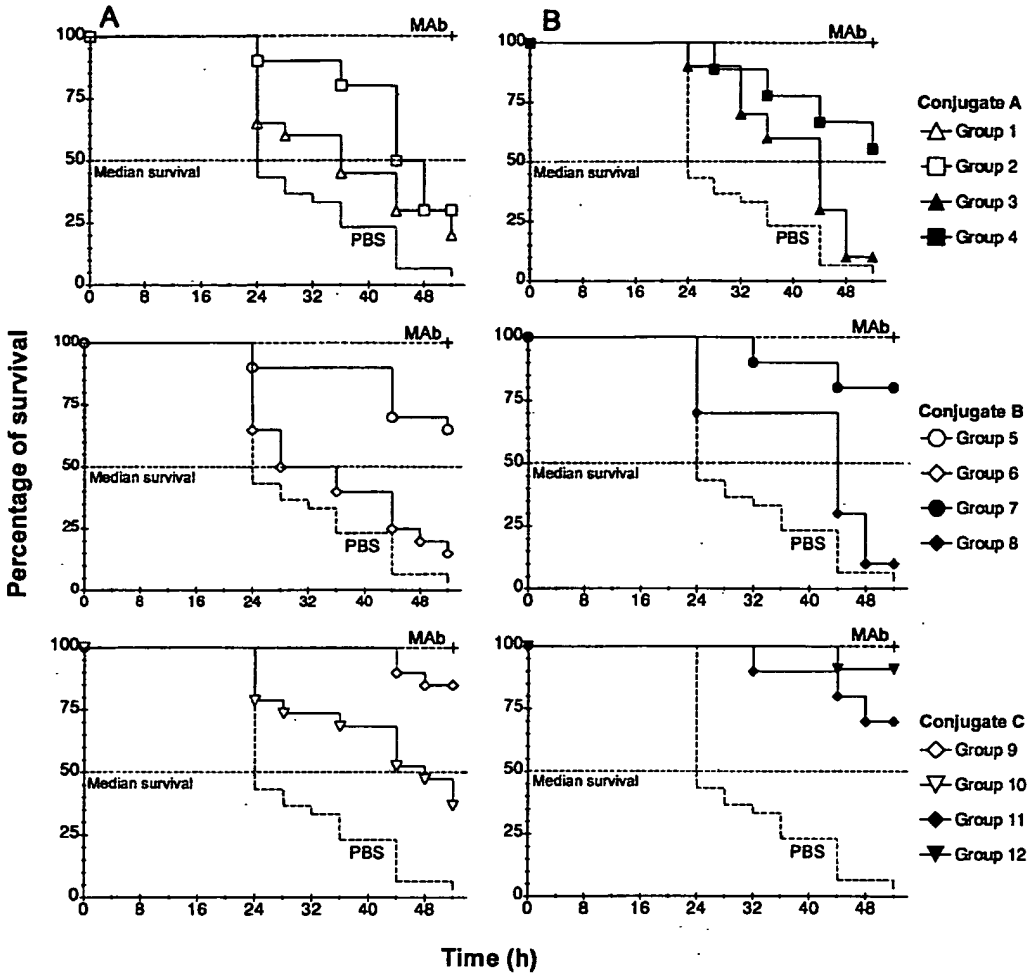

Figure 6. For an infant mouse protection assay, 4–5-day-old CD-1 mice were challenged with a mixture of pooled tertiary serum samples and virulent *Vibrio cholerae* O395 (29–43 LD_{50}). Mice were observed every 4 h for time to death, beginning at 24 h. *Top,* Mice that received conjugate A; *middle,* conjugate B; *bottom,* conjugate C. For a description of conjugates A, B, and C, see the section "CHO-BSA conjugates" in Materials and Methods. *A,* Mice that received CHO (Ogawa terminal hexasaccharide)–bovine serum albumin (BSA) without adjuvant. *B,* Mice that received CHO-BSA in adjuvant. Saline mixed with virulent challenge strain served as negative control *(dashed descending line).* Monoclonal antibody (MAb) A-20-6 combined with virulent challenge strain was used as a positive control (*horizontal dashed line,* indicating 100% protection). Average survival was calculated for each group at 52 h and was used to generate the *X*-axes for figure 7. Comparison of survival curves for conjugate A vs. conjugate B, $P = .17$; for conjugate A vs. conjugate C, $P < .0001$; for conjugate B vs. conjugate C, $P = .002$ (log-rank test).

Discussion

Cholera is an enteric disease that can reach epidemic proportions. One factor that can moderate a cholera outbreak is the immune status of the population at risk. The currently available vaccines are not universally effective for all target populations, particularly very young children. LPS is a candidate antigen for a cholera subunit vaccine. Anti-LPS IgM is thought to be operative in in vitro vibriocidal assays in which a positive response correlates with clinical protection in humans [3]. Which isotype it is most important for cholera vaccines to induce is a source of contention. It is thought that IgA and IgG, rather than IgM, play a role in protection against cholera [1, 4, 6, 7, 14]. Although IgA or IgG can be induced by *V. cholerae* LPS, the difficulty of generating these generally T cell–dependent antibodies may contribute to cholera vaccine failure rates.

Carbohydrate- or LPS-protein conjugates have been generated and tested. The putative protective carbohydrate epitopes are directly conjugated to a protein carrier so that a T cell–independent response will be altered to a T cell–dependent response, which can lead to class switching, increased affinity of the specific antibodies, and B cell memory [1, 16, 39, 40]. Vaccines currently

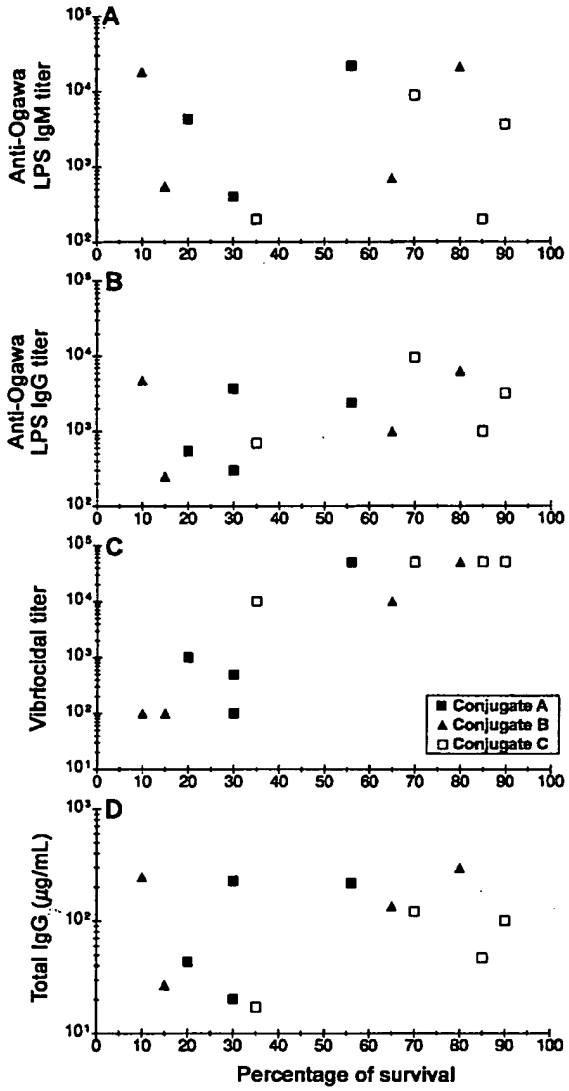

Figure 7. Correlations between ELISA IgM titer (*A;* conjugate A, $r^2 = 0.78$; conjugate B, $r^2 = 0.03$; conjugate C, $r^2 = 0.05$), IgG titer (*B;* conjugate A, $r^2 = 0.15$; conjugate B, $r^2 = 0.10$; conjugate C, $r^2 = 0.03$), vibriocidal titer (*C;* conjugate A, $r^2 = 0.90$; conjugate B, $r^2 = 0.70$; conjugate C, $r^2 = 0.88$), and concentration of serum *Vibrio cholerae* Ogawa–specific IgG (*D;* conjugate A, $r^2 = 0.39$; conjugate B, $r^2 = 0.18$; conjugate C, $r^2 = 0.35$) and percentage of protection. For a description of conjugates A, B, and C, see the section "CHO-BSA conjugates" in Materials and Methods. All comparisons were based on pooled tertiary antiserum samples. Protection correlated most closely with vibriocidal activity of antiserum, as shown by the distribution in *C* and the reported correlation coefficients. (In panel *A*, 2 different groups received conjugate A and had titers of 1:400 and protection levels of 30%; thus, only 3 solid squares are seen, because 2 squares overlap.)

in clinical use apply this strategy and provide protection that is enhanced in comparison with the protection provided by immunization with the unconjugated antigens [20]. LPS is toxic in its native form but is still a potent immunogen. LPS-specific B cell hybridomas have been made that secrete antibodies characteristic of T cell–dependent isotypes IgA, IgG1, and IgG3 [7, 37].

In an attempt to generate better O-SP–based immunogens for cholera, 2 groups have used whole *V. cholerae* O1 Inaba or O139 LPS that was detoxified and conjugated to either CT [1] or tetanus toxoid [16]. The typical immunization regimens of these groups used microgram amounts of the conjugate, sometimes emulsified in adjuvant, which was given subcutaneously 3 times at 2-week intervals. Gupta et al. [1] reported low IgM titers (geometric mean, 30–130) after 3 immunizations and no IgG response until after the third immunization, 35 days after primary inoculation with CT-conjugated Inaba LPS adsorbed on alum hydroxide. The vibriocidal activity of the tertiary antiserum described by Gupta et al. [1] ranged from 1:1000 to $1:1 \times 10^6$. In our study, the IgM and IgG ELISA titers were typically 1–2 orders of magnitude higher, and the responses were evident at an earlier point (+10 and +17 days) in the immunization scheme. The vibriocidal titers reported for both studies (the present study and [16]) were of similar magnitude. The protective capacity of Inaba antiserum after immunization of mice with detoxified LPS conjugated to CT was not reported by Gupta et al. [1].

In this study, mice that received conjugate C (molar ratio of CHO to BSA, 4.6:1) produced more-protective antiserum (70% protective) than did mice immunized with conjugate A (molar ratio of CHO to BSA, 15.5:1; 34% protective) or conjugate B (molar ratio of CHO to BSA, 9.2:1; 43% protective). The reasons for this are unclear. The IgG and IgM ELISA titers of the tertiary antiserum samples were similar for all groups that received CHO-BSA in adjuvant but did not correlate well with protection (figure 7*A* and 7*B*). Others, who have investigated anti–pneumococcal polysaccharide responses, have reported that ELISA titers do not correlate with the titers of a test that assesses the protective nature of the antiserum [41]. These authors postulated that, in their system, the ELISA demonstrated cross-reactive antibodies that bound the antigen but that were ineffective at binding it to produce a protective effect. This is complicated by the fact that ELISA titers reflect the affinity of the antiserum for the antigen, as well as the concentration of the specific antibody. Thus, antigen-reactive but not functional antibodies in a given serum sample can dominate the ELISA. These antibodies would obscure the contribution of functional antibodies that are not present in a high concentration if the functional antibodies do not react with a similar affinity; yet, the functional antibodies may be highly effective in responding to the antigen expressed in situ on the infectious organism.

There are at least 2 potential experimental reasons for the differential protection that we measured in the protection assay: (1) the lower level of substitution of the CHO on the carrier for

conjugate C and (2) the lower number of immunizations with conjugate C. The most-protective antiserum was from mice exposed to less antigen (measured by total mass and degree of substitution on the carrier). A likely explanation for the differences in protective capacity of the antiserum is that certain elements associated with individual antibody generation and/or selection define the protective capacity of the antiserum. These elements include preferred heavy- and light-chain pairing, junctional diversity, and somatic mutation. One desired outcome of T cell–dependent humoral response is improvement of the binding characteristics (higher affinity) of the antibodies by somatic mutation of the variable regions. In the human response to the *Haemophilus influenzae* type b vaccine, a preferred light-chain variable region that has an additional residue in the complementarity-determining region 3 is associated with protection [17]. The antigen dose or the use of adjuvants can affect the development of protective titers to LPS [42–44]. Immunization studies with a high–molecular-weight O-SP of *Pseudomonas aeruginosa* indicated that low doses were more immunogenic than high doses [45]. Other studies of anti–*P. aeruginosa* LPS antibodies indicate that vaccinated, rather than chronically colonized, persons have antibody with higher affinity for O-chain epitopes [46]. Given the relatively restricted nature of carbohydrate epitopes in general, or the Ogawa epitope in particular, how many mutations or what type of mutations can be acquired is an open question. In the anti–*H. influenzae* type b O-SP antibody response, the germline configuration of the antibodies does not vary significantly, even when the O-SP is attached to a protein carrier [47].

The differences in the protective capacity of the anti-Ogawa antiserum from mice immunized with conjugates A or B and antiserum from mice immunized with conjugate C prompted us to reexamine the crystal structure of the anti-Ogawa MAb–perosamine complex [11] in the context of the cDNA sequences [9] of the selected anti-Ogawa MAbs. We compared the germline sequences of 2 members of the 7183 V family (7183.14 and 81X [35, 36]), which are likely the source of the heavy-chain variable gene segment expressed by the anti-Ogawa MAbs (figure 3). In addition to having many amino acids in common, as would be expected of family members, the 3 anti-Ogawa MAbs, like 7183.14 and 81X, all contain an Asp at position 33, which is critical for binding the terminal perosamine residue of *V. cholerae* O1 Ogawa O-SP [11]. The other members of the 7183 family, some 17 MAbs, would have to have a mutation at position 33 to Asp, as well as in various other residues, to match the sequence of the Ogawa-specific MAb heavy chain.

If the amino acids at other positions in the sequences in figure 3 are examined for differences, it is apparent that substitutions at position 5 (Glu), 13 (Thr), and 19 (Arg) are not seen in the 2D6 MAb and germline sequences, although these substitutions are present in the S-20-4 and A-20-6 series. The locations of these changes on the crystal structure indicate that the changes are not within the hypervariable site (data not shown). Residues 27 and 28 on the heavy chain may be important to the specificity of anti-Ogawa MAbs but are mutated or not in these positions depending on which germline V is chosen for comparison to a particular MAb heavy-chain sequence. This variability and the relative position of amino acids 27 and 28 on the crystal structure, some 15 Å from Asp 33, are not predictive of an important function. There are other mutations in S-20-4 (Arg56, Asp73, Gln87), compared with the 7183.14 or 81X sequences, but, in the crystal model, they are distant from the combining site, compared with Asp 33.

The crystal structures of the complex of antibody S-20-4 with the monosaccharide and the disaccharide [11] show that carbohydrate-binding affinity is the result of 2 basic contributions: the formation of 6 intermolecular hydrogen-bonding interactions and the burial of nonpolar groups at the interface. Four of the 6 hydrogen bonds are formed between the hydroxyl groups of the terminal sugar residue and 2 charged amino acid residues from the heavy chain, Asp33 and His99. These 2 residues are clearly important to defining the antibody specificity and are conserved in known anti-Ogawa antibodies. Another important feature revealed by the crystallographic study of S-20-4 is a patch of hydrophobic side-chain residues at the center of the antibody interface. This patch (figure 3) is formed by residues from both the light chain (Tyr34, Trp93, Trp98) and the heavy chain (Phe50, Tyr101, Ala102). In particular, these residues define a nonpolar pocket that accommodates the 2-*O*-methyl group of the terminal perosamine residue, which is critical for antigen recognition [9, 11].

Our analysis indicates that the amino acids Phe50 (same in MAb sequence and crystal numbering) and Ser59 (Ser58, in Kabat numbering) are mutated, compared with all but 1 of the germline sequences [35, 36]. In the 7183 V family germline, the translated amino acids for these 2 positions are typically a Thr50 and a Tyr59, which are significantly different in side-chain bulk and potential for hydrogen bonding from the Phe50 and Ser59 residues present in the anti-Ogawa MAbs. Inspection of the S-20-4 crystal structure suggests that these amino acid substitutions are consistent with those selected by the antigen. The 2 residues are close to Asp33 at the center of the interface; one of them (Phe50) is directly involved in the hydrophobic interface, and the other (Ser59) forms hydrogen-bonding interactions with Gly96 at the tip of the third hypervariable region of the light chain. This last interaction might be important to stabilization of the conformation of the L3 loop (WYSGHW), which contains 2 tryptophan residues important for antigen binding. Furthermore, the 2 substitutions could be correlated, because the presence of a germline tyrosine at position 59 (as observed, for example, in the 3-dimensional structure of the anti-peptide antibody 17/9, PDB code 1HIN) would sterically clash with Phe50.

Somatic mutations of antibody variable regions may be present and yet not have a positive effect on the binding characteristics of the antibody. Somatically mutated anti-CHO antibodies do not necessarily have an advantage over germline configuration antibodies [17]. The significance of amino acid changes in anti-Ogawa MAb relative to germline is not known. However, our model, which is based on the relationship between the role of

the mutated residues and their location in the crystal structure, fits well with the established paradigm, that a low concentration of antigen can select somatically mutated antibodies that function "better" than the germline sequences. In our study, mice immunized with conjugate C produced the most-protective antibody. These mice received fewer immunizations with conjugate C, the conjugate with the lowest substitution of CHO per BSA. Future studies are needed to determine how the level of CHO substitution and number of immunizations affect the repertoire profile of the anti–Ogawa epitope response, but regulation of these parameters clearly has a large impact on the protective capacity of the antiserum.

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